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13. Abstract (Maximum 200 Words) <i>(abstract should contain no proprietary or confidential information)</i> Breast cancer arises through the accumulation of genetic alterations that affect two classes of genes, oncogenes and tumor suppressor genes. These genes must be identified for several reasons. Characterization of the genes that drive carcinogenesis will facilitate a better understanding of cancer development. As part of this big picture, we have studied tumor suppressor genes involved in breast cancer. A tumor suppressor candidate, DBC2 was analyzed. DBC2 is a structurally new gene and its function remains to be studied. DBC2 is composed of a RAS domain, protein-protein interacting domains, and DNA binding domain. DBC2 expression is extinguished in more than half of breast tumors we tested. Methylation analysis of tumor cells revealed hypermethylation of the CpG islands in the DBC2 promoter region. Additionally, we have discovered somatic mutations of DBC2 in breast cancer. These mutations are accompanied by LOH. When DBC2 expression was induced in tumor cells, the cell growth was hindered. In contrast naturally occurring mutants did not suppress growth of the tumor cells. Our findings suggest that DBC2 is the target of mutations at 8p22.			
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Introduction

In the course of carcinogenesis, cells acquire a number of genetic changes that are recorded in their genomes, most of which have no effect on cellular function. There are, however, critical genetic alterations that affect two classes of genes: oncogenes that are activated or altered in function; and tumor suppressor genes that are down-regulated or abolished in cancer cells. These alterations include amplifications, deletions, insertions, translocations, single nucleotide substitutions, hypermethylation, and chromosomal abnormalities. Discovery and analysis of these genetic changes have contributed to a better understanding of the molecular basis of cancer development. We have discovered homozygously deleted loci that may contain new tumor suppressor genes. Isolation and characterization of genes from these loci may lead to a better understanding of tumorigenesis and more effective diagnosis and treatment of cancer.

BODY

Approved Statement of Work

Task 1. To narrow down the commonly deleted region in breast tumors (months 1-6)

- ◊ generate STS markers in the deletion epicenter (months 1-2)
- ◊ perform deletion mapping of tumor pairs (months 3-6)
- ◊ identify the breakpoint in each tumor cell (months 5-6)

Task 2. To construct a transcript map of the commonly deleted region (months 1-18)

- ◊ identify transcripts from the region (months 1-9)
- ◊ isolate full-length cDNA (months 7-18)

Task 3. To characterize the genes affected by the deletion (months 19-30)

- ◊ screen expression pattern of the genes (months 19-24)
- ◊ search for mutations of the genes in breast tumors (months 25-30)

Task 4. To study the biology of candidate genes for tumor suppressor (months 30-48)

- ◊ make antibody of the gene product (30-36 months)
- ◊ examine the changes of phenotype by the induction of the genes (30-36 months)
- ◊ RNA profiling of the cells before and after the induction (30-36 months)
- ◊ determine localization of the gene product (37-39 months)
- ◊ Examine biological function of the gene product (30-48 months)

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Task 1

Task 1 was completed in previous years. The following is a brief summary of the outcome.

Characterization of the deletion locus

We have discovered homozygously deleted regions in breast cancer using RDA (Representational Difference Analysis). Among them, a deletion at human chromosome 8p21 was investigated further. Sixty STS's mapped to the locus were used to construct physical and deletion maps. The commonly deleted region was narrowed down to approximately 200 kb.

LOH Analysis

LOH study was also performed. Of tumors from the 73 patients informative for D8S1734, 20 (27 percent) had lost one allele at the marker.

Task 2

Task 2 was completed in previous years. The following is a brief summary of the outcome.

Isolation of Transcripts from 8p21 Locus

Seven genes were isolated from the commonly deleted region. They were TNFRSF10B, TNFRSF10A, EGR3, BIN3, KIAA0273, DBC1 and DBC2 (accession number AY009093). The full length cDNA of DBC1 and DBC2 were registered to GenBank (Accession numbers AF293335 and AY009093, respectively)

Task 3

Task 2 was completed in previous years. The following is a brief summary of the outcome.

Mutation Analysis

We did not detect a significant mutations in DBC1 or TNFRSF10B by DHPLC (Denatured High Performance Liquid Chromatography) and SSCP (Single Strand Conformation Polymorphisms) analyses. In contrast we discovered two somatic mutations in breast cancer specimens and two additional missense mutations in lung and breast cancer cell lines. In total, from cell lines and tumors, four missense mutations in the coding region of DBC2 were found.

Computational analysis of DBC2

To the best of our knowledge, DBC2 is a previously unreported gene and a member of a RAS-BTB gene family.

Expression Studies

Expression studies of DBC2, DBC1, and TNFRSF10B were performed by RT-PCR and RNA blotting. We find that all three genes are expressed ubiquitously in all normal sources. However, DBC2 expression is extinguished in about half of breast and lung cancer specimens, but not in colon or other cancers we examined. In contrast, neither TNFRSF10B nor DBC1 expression is substantially extinguished in cancers from any source.

Task 4

Subcellular localization of DBC2

This was completed in previous years. Using DBC2-green fluorescent protein (GFP), we demonstrated that DBC2 was localized in the nucleus.

Inducible Gene Expression System

To study effects of DBC2 induction in breast cancer cell lines lacking the DBC2 transcript, we utilized an inducible gene expression system (1). Four retroviral vectors (a control, a gene transfer, and two receptor vectors) are used in the retrovirus-mediated ecdysone inducible gene expression system. When the host cell line is treated with an ecdysone analog, muristerone A, the receptors form complexes and activate ecdysone responsive elements (E/Gre). The activated E/Gre promotes transcription of a gene under a minimal heat shock promoter. To establish stable host cell lines, several cell lines including 293, HeLa and T-47D were transfected with retroviral vectors with ecdysone receptors. We then transformed the host cells with E/Gre-GFP and analyzed them by a fluorescent activated cell sorter (FACS) without induction in order to eliminate cells with leaky expression of the transgene. The remaining cells were treated with muristerone A and then analyzed by FACS. The GFP-positive cells were collected and verified by microscopic observation. The selected host cells had no detectable expression of GFP before induction but, after induction, had substantial expression of GFP.

DBC2 Induction in Breast Tumors

We previously demonstrated that induction of DBC2 had no effect on growth of HeLa cells, which expressed endogenous DBC2. In contrast, growth of T-47D cells was greatly hindered by DBC2 induction (Figure 1). The next question was if mutated DBC2 could also suppress tumor growth. We generated mutant DBC2 fragments containing the same missense mutations we discovered in breast cancer specimens. Induction of the mutants did not show growth inhibition (Figure 1). The cancer cells with mutated allele have LOH of DBC2 locus, indicating absence of wild type DBC2. These findings indicate that DBC2 is the target of the genetic alterations at 8p22 (2).

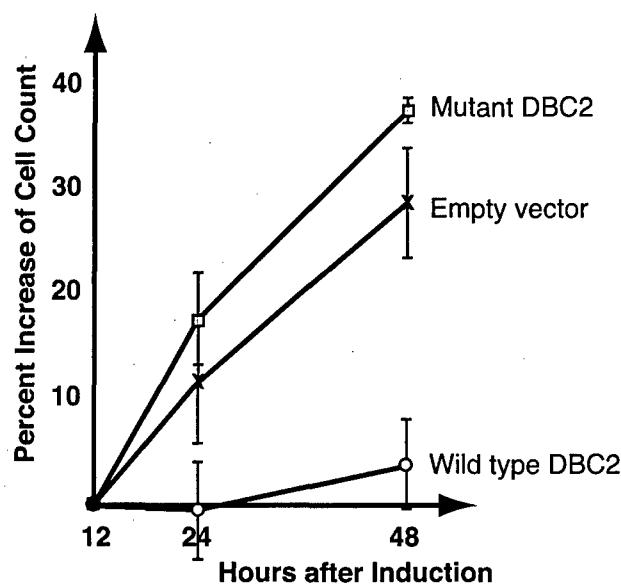


Figure 1. Growth curve of T-47D. T-47D with various constructs were treated with Muristerone A. Induction of the target genes was confirmed by RNA blot analysis. Growth curves of each cell lines were drawn at several time point after induction. Percent increase of the cell count from 12 hours after induction is shown. Expression of wild type DBC2 inhibits cell growth of T47D while mutant DBC2 and the empty vector does not.

RNA profiling

We are currently analyzing expression patterns of T-47D using expression microarrays containing 46,000 probes. The acquired data are statistically processed with a computer program (S-Plus) and alterations of gene expression level are examined. RNA profiles of T-47D cells with inducible DBC2 are compared with those with an empty vector. We will look for genes whose expression level is changed after DBC2 induction by more than three folds

Biological function of DBC2

To analyze functional domains of DBC2, we performed a framework. Since the BTB domain is predicted to serve as a protein-protein interacting domain, isolation of proteins that interact with DBC2 is important. We will utilize yeast two hybrid systems and immunoprecipitation to achieve this goal. We have constructed four two-hybrid libraries and DBC2 constructs for the system. The RAS domain shows significant homology to known G-proteins. Since some mutations are found to alter the function of RAS protein, we will make equivalent mutations in the RAS domain and functionally analyze them. Constructs containing these mutations have been generated by site-directed mutagenesis.

Future experiments

We will make a catalog of genes whose expression is significantly altered by expression microarray analysis. We will isolate proteins that are interacting with DBC2 through the BTB domain. Once interaction is confirmed, mutations will be introduced in the BTB domain and the interaction will be examined. Effects of mutagenesis in the RAS domain will be studied.

Key research Accomplishments

- Studies on effects of wild type DBC2 induction in tumor cells
- Studies on effects of mutant DBC2 induction in tumor cells.
- Preparation for functional analysis of DBC2 including expression microarray, mutagenesis, and cDNA library construction.

Reportable Outcomes

Publication

Hamaguchi M., Meth J. L., von Klitzing C., Wei W., Esposito D., Rodgers L., Walsh T., Welch P., King M. C. and Wigler M. H., "DBC2, a candidate for a tumor suppressor gene involved in breast cancer." *Proc Acad Natl Sci USA* **99**: 13647-13652, 2002.

Conclusions

A tumor suppressor candidate, DBC2 was analyzed. DBC2 is a structurally new gene and its function remains to be studied. DBC2 is composed of a RAS domain, protein-protein interacting domains, and DNA binding domain. DBC2 expression is extinguished in more than half of breast tumors we tested. Methylation analysis of tumor cells revealed hypermethylation of the CpG islands in the DBC2 promoter region. Additionally, we have discovered somatic mutations of DBC2 in breast cancer. These mutations are accompanied by LOH. When DBC2 expression was induced in tumor cells, the cell growth was hindered whereas mutated DBC2 failed to suppress growth of the tumor cells. Our findings suggest that DBC2 is the target of genetic alterations at 8p22. We will continue studying DBC2 and biological pathways it belongs to so that the better understanding of breast cancer development can be facilitated.

References

1. Stolarov, J., Chang, K., Reiner, A., Wigler, M., and Mittal, V. (2001) *Proc Natl Acad Sci U S A* **98**, 13043-1304.
2. Hamaguchi, M., Meth, J. L., von Klitzing, C., Wei, W., Esposito, D., Rodgers, L., Walsh, T., Welcsh, P., King, M. C. and Wigler, M. H., "DBC2, a candidate for a tumor suppressor gene involved in breast cancer." *Proc Acad Natl Sci USA* **99**: 13647-13652, 2002.

DBC2, a candidate for a tumor suppressor gene involved in breast cancer

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Contributed by Michael H. Wigler, August 26, 2002

A previously uncharacterized gene, *DBC2* (*deleted in breast cancer*), was cloned from a homozygously deleted region at human chromosome 8p21. *DBC2* contains a highly conserved RAS domain and two putative protein interacting domains. Our analyses indicate that *DBC2* is the best candidate tumor suppressor gene from this region. It lies within the epicenter of the deletions and is homozygously deleted in 3.5% (7/200) of breast tumors. Mutation analysis of *DBC2* led to discovery of two instances of somatic missense mutations in breast tumor specimens, whereas no missense mutations were found in other candidates from the region. Unlike other genes in the region, expression of *DBC2* is often extinguished in breast cancer cells or tissues. Moreover, our functional analysis revealed that *DBC2* expression in breast cancer cells lacking *DBC2* transcripts causes growth inhibition. By contrast, expression of a somatic mutant discovered in a breast cancer specimen does not suppress the growth of breast cancer cells.

In the course of carcinogenesis, cancer cells acquire a number of genetic changes, many of which may have no effect on cellular function. There are, however, critical genetic alterations that affect two classes of genes: oncogenes that are activated or altered in function and tumor suppressor genes that are down-regulated or ablated in cancer cells. Discovery and analysis of these genetic changes have contributed to a better understanding of the molecular basis of cancer development. Studies on *BRCA1* and -2 tumor suppressor genes have provided useful information for understanding familial breast cancer (1). Other tumor suppressor genes are involved in sporadic breast cancer, including *PTEN* and *p53* (2, 3). However, it is likely that many, if not most, tumor suppressor genes responsible for sporadic breast cancer remain to be discovered. Representational difference analysis (RDA) is one tool that has been shown to be effective in the hunt for tumor suppressor genes (3, 4). We have analyzed a few breast cancer biopsies by RDA and isolated a number of deletion probes. An RDA probe detecting homozygous deletion was mapped to human chromosome 8p21, and we find this region to be deleted in other breast cancer specimens as well. We have mapped this region, cloned new genes residing therein, and have found somatic mutations in cancer cells for one of the genes from this locus.

Materials and Methods

Materials. Breast tumor specimens were procured from a variety of sources. Aneuploid and diploid nuclei from breast tumor specimens were prepared by fluorescence-activated sorting by using an Elite EPS (Beckman Coulter). DNA from the sorted nuclei was immortalized by making *Dpn*II and *Bfa*I representations, by using methods described elsewhere (5). Cell lines were purchased from American Type Culture Collection or are from our permanent collection inherited from Jorgen Fogh (Memorial Sloan-Kettering Cancer Center). Chemicals and buffers were obtained from Sigma unless otherwise noted. Enzymes were obtained from New England Biolabs except *Taq*DNA Polymerase (AmpliTaq), from Perkin-Elmer. All enzymatic

reactions were performed with buffers recommended by the supplier.

Deletion Analysis. Deletion mapping was performed by quantitative PCR (Q-PCR) with the ABI Prism 7700 Sequence Detector (6). The Q-PCR was carried out in three stages: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. DNA markers from 8p21 and other chromosomes were used for Q-PCR. DNA markers were derived from the original RDA probe WD31; from sequence tag sites WI-14743, WI-15206, and WI-16696; from genes we discovered [*deleted in breast cancer 1 (DBC1)* (GenBank accession no. AF293335) and *deleted in breast cancer 2 (DBC2)* (accession no. AY009093)]; and from known genes from this region, including *EGR3* (accession no. NM004430), tumor necrosis factor receptor super family 10B (*TNFRSF10A*) (accession no. AF014794), *TNFRSF10B* (accession no. AC007868), and *KIAA0273* (accession no. NM014759). Additionally, DNA probe ER48 was a subcloned fragment from a genomic clone from human chromosome 3p14, and WB23 was a cloned fragment from human chromosome 21p.

The protocols for RDA are described elsewhere (4). All oligonucleotides were synthesized by Operon Technologies (Alameda, CA), except that Taqman probes for Q-PCR were synthesized by Perkin-Elmer with 6-carboxyfluorescein at the 5' end and 6-carboxy-tetramethyl-rhodamine at the 3' end.

Gene Cloning. Gene discovery methods included exon trapping (7), rapid isolation of cDNA by hybridization (8), cDNA library screening, and sequence determination of genomic clones followed by database searches. RNA ligase-mediated amplification of cDNA ends (RLM-RACE) was performed by using the First Choice RLM-RACE kit (Ambion, Austin, TX) (9). Computational analyses of the fragments were performed by BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST), GCG Wisconsin Package, and a PFAM HMM search (10, 11).

Mutation Analysis. Mutational analyses were performed by a protein truncation test (PTT), denatured HPLC (DHPLC), and single-strand conformation polymorphism (SSCP). For PTT, the entire ORF of *TNFRSF10B* was amplified with primers, TAATACGACTCACTATAGGGAGCCACCATGGAACAA-CGGGGACAG and AATTGTGGCACTTCCTACTGACT. The PTT procedure is described elsewhere (12). WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE) was used for DHPLC (13). The target fragments were amplified by PCR with the GeneAmp PCR System 9600 (Perkin-Elmer). Control fragments were generated by PCR from normal human

Abbreviations: DHPLC, denatured HPLC; HD, homozygous deletion; Q-PCR, quantitative PCR; SSCP, single-strand conformation polymorphisms; RDA, representational difference analysis.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY009093 (human *DBC2*), AF293335 (human *DBC1*), and AF420001 (mouse *DBC2*)].

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DNA. The samples and control fragments were mixed, denatured, reannealed to generate heteroduplexes, and then analyzed by DHPLC. The samples with different peak patterns were sequenced. Protocols for SSCP are described elsewhere (14). The primer sequences for Q-PCR, DHPLC, and SSCP used in this study will be provided on request.

Expression Analysis. Expression analyses were mainly done by RT-PCR. Cytoplasmic RNA was extracted from cultured cells, and total RNA was extracted from tissue samples with guanidinium thiocyanate followed by centrifugation in cesium chloride solutions. The protocols are described elsewhere (15). RNA of normal tissues was either purchased from BD Biosciences (Palo Alto, CA) or extracted from surgical specimens. Avian myeloblastosis virus reverse transcriptase purchased from Boehringer Mannheim was used to synthesize cDNA with oligo-dT primers. PCR was then performed with AmpliTaq. The 50- μ l PCR reaction mixture contains 1 μ l of the cDNA, 200 μ M of each dNTP, 1 μ M of each primer, 2 units of AmpliTaq, and 1 \times AmpliTaq buffer. After initial denaturation at 94°C for 10 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 3 min were performed. The primer sequences used in this analysis were: AACATGGAGGCCCTGTGCCAGCAGAA and ACTCGCAGCTCCTGCATCTCT (*DBC1*); ACCATGTGG-TACCCAGAAATC and TTAGACAACAGCAGCAGGGT-CAGA (*DBC2*); AGTTTGACCAGAGATGC and TGAC-CAAGGCTGAATAATCCC (*TNFRSF10A*); CTTGATTGT-GGCTGTGTTGTT and GCCACCTTATCTCATTGTCCA (*TNFRSF10B*); AGCATGGAGCTGCTGTCCAC and TTAGCT-CCCTGAACCTGGGCTTAC (*KIAA0273*); and CCATGGAG-GAGCCGAGTCAGA and TTAGTCTGAGTCAGGCCCT-TCTGT (*p53*).

Mutagenesis. Mutation was introduced by using the Exsite Mutagenesis kit (Stratagene). Sequences of primers used for mutagenesis were ATGAACCTGAGTGAGGGGGAGCT and CAGCTCCCCCTCACTCAGGTT. The generated mutation was confirmed by sequencing of both strands.

Gene Expression. HeLa and T-47D cells were grown in DMEM supplemented with 15% FCS. For constitutive gene expression, a mammalian expression vector pcDNA3.1/His (Invitrogen) was used. For inducible gene expression, an ecdysone gene expression system was used. Stable host cell lines carrying ecdysone receptors were established by using retroviral vectors (16). The genes of interest were cloned into pIND (Invitrogen) with an Xpress tag at the 5' end and transfected into the host cell lines. All constructs were verified by sequencing. The cells were transformed with 15 μ g of plasmid by electroporation (field strength = 0.25 kV/cm, capacitor = 950 μ F) using Gene Pulser II (Bio-Rad) and selected with G418 (400 μ g/ml) and Zeocin (Invitrogen) (150 μ g/ml). Muristerone A (Invitrogen) (5 μ M) was administered to induce the genes in pIND. Cell growth was monitored by using the CyQuant Cell Proliferation Assay kit (Molecular Probes).

Sequencing. DNA sequencing was performed with the ABI Thermal Cycler Sequencing kit and ABI Prism 377 DNA Sequencer (Perkin-Elmer). Both strands were sequenced unless otherwise specified.

Results

Homozygous Deletion. RDA of breast cancer samples generated a number of deletion probes, including WD31 from a tumor called BBr67. Homozygous deletion (HD) of WD31 in BBr67 was confirmed both by Southern blotting of the DNA representations made from the aneuploid nuclei of BBr67 and by the semiquantitative PCR analysis of genomic DNA made from

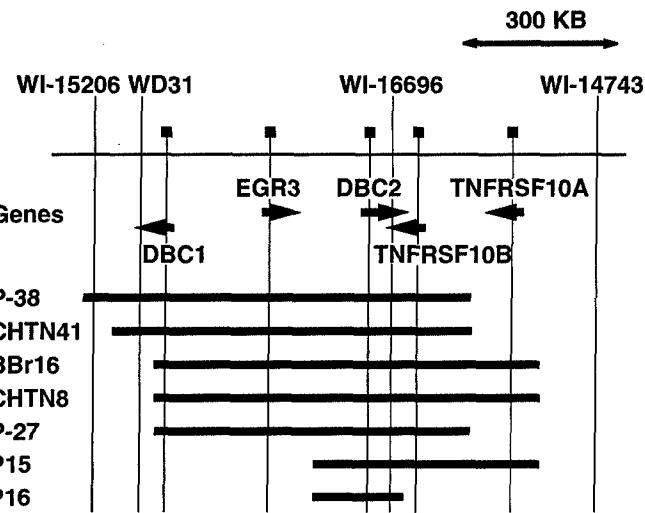


Fig. 1. Deletion map of 8p21. The vertical lines indicate the localization of sequence tag sites and genes used for deletion mapping, all of which were tested against 200 tumor samples. Genes are represented by a horizontal line with an arrowhead. The STs are listed above the line, and the lines with a square indicate probes derived from the genes. The arrow with double heads indicates approximately 300 kb. Horizontal lines in the lower half represent deletions found in the tumor samples (named, Left). The *DBC2* probe and WI-16696 demonstrated the highest deletion frequency. Both *DBC2* and *TNFRSF10B* genes are overlapping with different orientations and deleted in all tumor specimens.

these nuclei. WD31 was mapped to 8p21 by radiation hybrid panels and genomic library screening, and the locus was scrutinized further. We constructed a contig of genomic clones and applied positional cloning methods, described in *Materials and Methods*. We discovered two previously uncharacterized genes and identified four known genes extending outward from WD31: *DBC1*, *DBC2*, *TNFRSF10A*, *TNFRSF10B*, *EGR3*, and *KIAA0273*. These genes were mapped by us, and our mapping order was confirmed by comparison to the human genome sequence (www.ncbi.nlm.nih.gov/) (Fig. 1).

Deletion Analysis. These genes, as well as nearby sequence tag sites, were then used for deletion analysis. Aneuploid and diploid nuclei from breast cancer were sorted and analyzed to determine deletion frequency and the deletion epicenter. Although aneuploid nuclei will be enriched for tumor cells, and diploid nuclei will be enriched for normal stroma, each fraction will be contaminated to varying extents with each other. Real-time PCR or Q-PCR was used to analyze genetic loss for markers in the region. Even in the presence of low levels of contamination, we can detect a homozygous deletion as a cycle difference between aneuploid and diploid samples (Fig. 2). We used multiple control markers for normalization, from chromosome 8 and other chromosomes. For example, we used ER48 and WB23 (from other chromosomes) to standardize the DNA amount and markers on the short arm of chromosome 8 to control for chromosome 8 copy number. In all cases tested, Q-PCR results were confirmed by Southern blotting of representations made from diploid and aneuploid nuclei (Fig. 2). The deletion mapping using 200 breast tumors revealed that the deletion frequency was 3.5%, and the deletion epicenter lay around a DNA marker WI-16696 that was surrounded by two genes. WI-16696 is contained in the intron 7 of *DBC2* and located within 1 kb of the 3' end of *TNFRSF10B*. On the basis of the transcription and deletion mapping (see Fig. 1), these two genes resided at the deletion epicenter, and hence we studied them further.

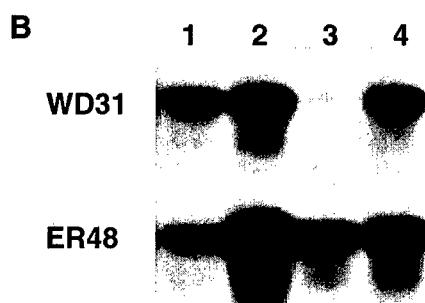
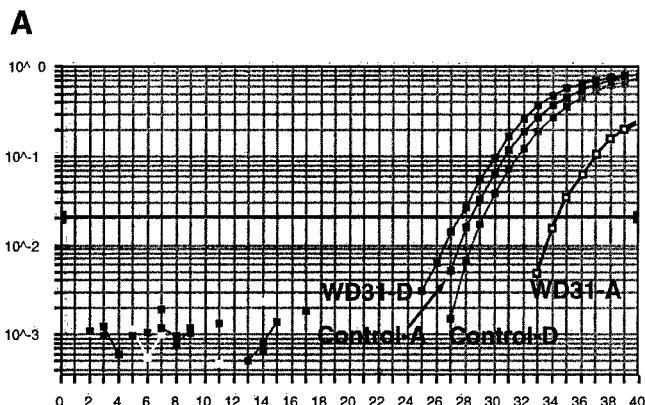


Fig. 2. Deletion analysis of tumor samples. (A) Real-time PCR analysis of a breast tumor sample CHTN41 is demonstrated. The abscissa is cycle number of PCR and the ordinate is quantity of PCR products. The control primer set in this figure is WB23 from chromosome 22. "D" and "A" represent DNA from diploid and aneuploid fractions, respectively. The amplification curve from aneuploid DNA with WD31 primer set has a several cycle difference from the others, indicating deletion of WD31 in this tumor. (B) Southern blot analysis of *Dpn*II representation with WD31 probe is shown. Each lane contains 5 μ g of *Dpn*II restriction fragments. Lanes 1 and 2 are DNA from a tumor and a matched normal (CHTN40), respectively, showing no deletion. Lanes 3 and 4 contain DNA from a tumor and a matched normal pair (CHTN41), showing deletion. ER48 is a control probe derived from chromosome 3. Lane 3 has a very faint band for *DBC2*, whereas the control probe exhibits the same intensity for tumor and normal samples, representing homozygous deletion. Contamination of normal stromal cells is considered to contribute the faint band.

Mutation Analysis. We began our study with *TNFRSF10B*, which encodes the Trail2 receptor for TNF, because mutations in the death domain of *TNFRSF10B* in nonsmall cell lung cancer had been previously reported (17). Mutation analysis of the entire ORF of *TNFRSF10B* was performed on 60 cancer cell lines by both protein truncation test and DHPLC followed by sequencing of variants, but no frameshift, missense, or nonsense mutations were found (see Table 1).

The coding region of *DBC2* was also screened for mutations by DHPLC and SSCP, and variants further studied by sequencing, revealing two missense mutations in the BTB/POZ (broad-complex/tramtrack/bric-a-brac/poxvirus/zinc finger) domain, found in lung and breast cancer cell lines. Ninety-five primary tumors and matched normals were then screened by either DHPLC or SSCP for mutations in either of these two genes (see Table 1). Samples showing variants in retention time (DHPLC) or mobility shifts (SSCP) were sequenced, and tumors were compared with matched normals (Fig. 3). No frameshift, missense, or nonsense mutations were found in *TNFRSF10B*. However, two missense mutations within *DBC2* were again discovered within tumors but not in matched normals, indicating that somatic mutations had occurred. One of these mutations, caus-

Table 1. Frequency of codon-altering point mutations found in three genes in the deleted region

Gene	Materials	Protein truncation test	SSCP	DHPLC
<i>TNFRSF10B</i>	Cell lines	0/60	NA	0/60
	Primary tumor	NA	0/90	NA
<i>DBC2</i>	Cell lines	0/60	2/60	2/65
	Primary tumor	NA	2/65	NA
<i>DBC1</i>	Cell lines	0/60	NA	0/60
	Primary tumor	NA	NA	NA

NA, not applicable.

ing Asp-299 \rightarrow Asn, was in the BTB/POZ domain and was confirmed by an independent PCR amplification and sequencing reaction. This mutation was used for functional studies described below. In total, from cell lines and tumors, four missense mutations in the coding region of *DBC2* were found (see Fig. 4), but no significant mutations were found in *TNFRSF10B*.

Computational Analysis. To the best of our knowledge, *DBC2* is a previously unreported gene. The cDNA for *DBC2* was obtained by cDNA library screening and completeness confirmed by using RNA ligase-mediated amplification of cDNA ends. PFAM analysis of our sequence revealed *DBC2* contained a RAS domain at its 5' end and two BTB/POZ domains. The RAS domain of *DBC2* has high homology to that of small GTP-binding proteins (G proteins), including the sites for binding to, and hydrolysis of, GTP (Fig. 4). However, *DBC2* is distinct from the typical RAS family members. First, *DBC2* has additional functional domains. Second, *DBC2* lacks the C-terminal lipid-binding sites that anchor the typical family member to membranes. BLAST analysis revealed two human genes with a similar RAS-BTB/POZ structure: *KIAA0740* and *KIAA0878* (accession nos. AB018283 and AB020685, respectively), the first one with high homology, and several genes in other organisms including *Mus musculus* (accession nos. AF420001, AK005770, and AK006650), *Dro-*

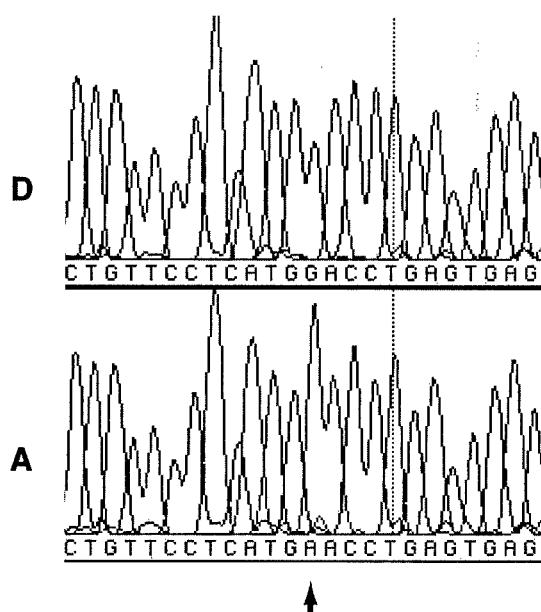


Fig. 3. Electrophoretogram of sequencing analysis. "D" and "A" designate sequencing results of DNA from diploid and aneuploid nuclei of tumor sample CHTN56, respectively. Arrow indicates position of somatic mutation.

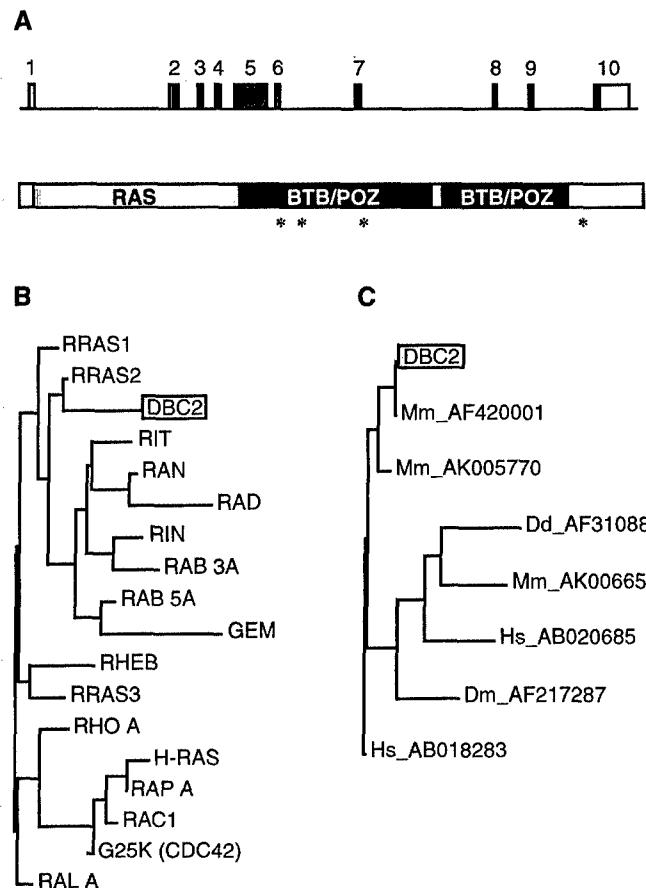


Fig. 4. Structure of *DBC2*. (A) The intron-exon structure of *DBC2* (GenBank accession no. AF315385) and predicted functional domains of *DBC2* are shown. The rectangles with a number represent exons. The shaded area indicates coding sequences. Based on deduced amino acid sequence, molecular mass of the predicted *DBC2* product is 83 kDa. The asterisks indicate missense mutations we have discovered. There were three in exon 5: T to G (7762), causing Tyr-284→Asp in a lung cancer cell line (SK-Mes-1), G to A (7807), causing Asp-299→Asn in a primary breast tumor (CHTN56), and A to C (8015), causing Asp-368→Ala in a breast cancer cell line (BT483); and one in exon 9: C to A (16389), causing Phe-647→Thr in a primary breast tumor (P-1). (B) Phylogenetic tree demonstrating overall homology between *DBC2* and human RAS family members is shown. A table of the pairwise distances between the genes was prepared by GCG Wisconsin Package, and a phylogram was then created from the table by using the neighbor-joining method. The length of a horizontal line is proportional to estimated divergence along each branch. Accession numbers of the genes used for this analysis are: *RRAS1* (P10301); *RRAS2* (P17082); *RIT* (AAB42213); *RAN* (P17080); *RAD* (P55042); *RIN* (AAB42214); *RAB3A* (P20336); *GEM* (P55040); *RHEB* (Q15382); *RRAS2* (Q14807); *RHO A* (P06749); *H-RAS* (P01112); *RAP A* (P10113); *RAC1* (P15154); *G25K* (P25763); and *RAL A* (P11233). (C) RAS-BTB proteins from different organisms are compared. Accession numbers of the genes are listed after the designation of the organism: Hs, *Homo sapiens*; Mm, *M. musculus*; Dm, *D. melanogaster*; and Dd, *Dictyostelium discoideum*.

sophila melanogaster (accession no. AF217287), and *Dictyostelium discoideum* (accession no. AF310886) (Fig. 4).

The BTB/POZ domain functions as a specific protein–protein interaction motif in known proteins (18). It is evolutionarily conserved and found primarily at the N terminus of zinc-finger transcription factors. Many BTB/POZ proteins are transcriptional regulators that mediate gene expression through the control of chromatin conformation; examples are *BACH1* and -2, *PATZ*, and *PLZF*, all of which interact with other proteins through their BTB/POZ domains. Unlike these proteins, *DBC2* has two BTB/POZ domains located at the C terminus.

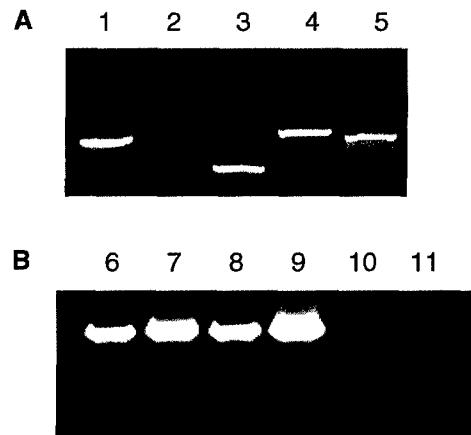


Fig. 5. RT-PCR analysis of tumor specimens. (A) RT-PCR products from mRNA of T-47D with five primer sets. Lanes 1–5 contain RT-PCR products with primers of *DBC1*, *DBC2*, *KIAA0273*, *TNFRSF10A*, and *p53*, respectively. Transcripts of all genes but *DBC2* were detected in T-47D. (B) RT-PCR products with the *DBC2* primer pair. Lanes 6–11 contain RT-PCR products from NCI-H82, Saos-2, SK-BR-3, SW 982, T-47D, and T84, respectively. *DBC2* expression was observed in NCI-H82, Saos-2, SK-BR-3, and SW 982 but not in T-47D or T84.

Expression Analysis. To gain more support for the hypothesis that loss of *DBC2* may play a role in breast cancer, and possibly other cancers, we examined its expression pattern in a variety of neoplastic and normal cells and tissues. We used RT-PCR to monitor the presence of *DBC2* mRNA, as well as two other genes from its locus, *DBC1* and *TNFRSF10B*. In a subset of cases, we tested our results by RNA blotting, and in all those cases, our RT-PCR results were confirmed. In Fig. 5, we show some representative data, illustrating the lack of expression of *DBC2* in T-47D, a breast cancer cell line. Our data are summarized in Table 2. For neoplastic cells and tissues, we drew on our collection of cultured tumor cell lines and frozen tissue biopsies from various sources, and for normal, we used multiple normal tissue samples, including breast and placenta (Materials and Methods). We find that all three genes are expressed ubiquitously in all normal sources. However, *DBC2* expression is extinguished in about half of breast and lung cancer specimens, but not in colon or other cancers we examined. In contrast, neither *TNFRSF10B* nor *DBC1* expression is substantially extinguished in cancers from any source.

Functional Analysis of *DBC2*. To study the function of *DBC2*, we expressed wild-type and mutated *DBC2* in cancer cell lines. We

Table 2. RT-PCR analysis of *DBC2*, *TNFRSF10B*, and *DBC1*

	Tissue of origin	<i>DBC2</i>	<i>TNFRSF10B</i>	<i>DBC1</i>
Neoplasm	Breast	42% (8/19)	89% (17/19)	84% (16/19)
	Lung	50% (7/14)	93% (13/14)	86% (12/14)
	Colon	83% (10/12)	100% (12/12)	92% (11/12)
	Other	91% (10/11)	82% (9/11)	100% (11/11)
Normal	Breast	100% (13/13)	100% (8/8)	100% (8/8)
	Lung	100% (1/1)	100% (1/1)	100% (1/1)
	Placenta	100% (2/2)	100% (2/2)	100% (2/2)
	Brain	100% (3/3)	100% (3/3)	100% (3/3)
Total		100% (19/19)	100% (14/14)	100% (14/14)

The percentage of the specimens with detectable expression of the indicated gene is listed. The denominator in parentheses indicates the number of specimens tested and the numerator, the number of positives. Neoplasm specimens are cell lines; normal specimens are tissue.

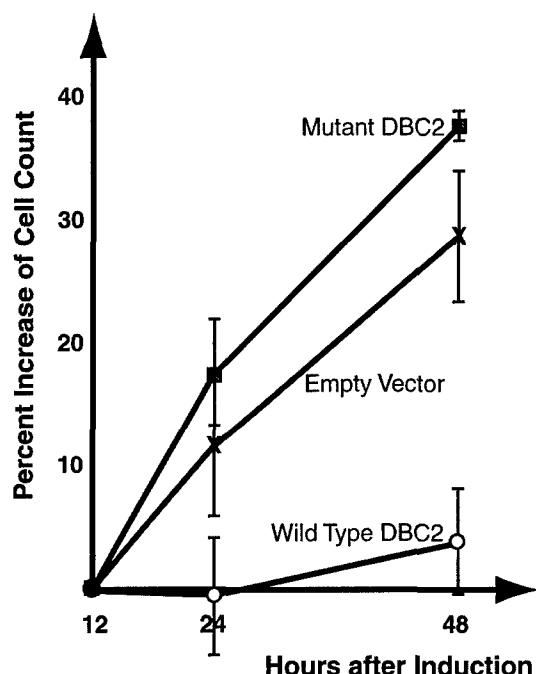


Fig. 6. Effects of gene induction on the growth of T47D. T-47D cells with various constructs were treated with 5 μ M Muristerone A. Percent increase of the cell count from 12 h after induction is shown. Expression of wild-type *DBC2* inhibits cell growth of T47D between 12 and 48 h after gene induction, whereas mutant *DBC2* and the empty vector have no influence on T47D proliferation.

generated the G-to-A mutation at nucleotide position 7807 (Asp-299 \rightarrow Asn), which was discovered in breast tumor specimen CHTN56.

First, wild-type *DBC2* and the mutant were cloned into pcDNA3.1/His for constitutive expression. Use of this vector results in expression of an epitope-tagged product. Wild-type and mutant *DBC2* vectors were introduced into HeLa cells that express endogenous *DBC2*. Expression of exogenous genes was verified by Western blot analysis, and neither affected the growth of the HeLa cells. Next we tested T-47D, the breast cancer cell line described above and in Fig. 5. T-47D cells transformed with wild-type *DBC2* were difficult to obtain, and even when transformants were cloned, expression of *DBC2* could not be detected.

We therefore used an ecdysone inducible gene expression system to analyze effects of *DBC2* expression in T-47D, using pIND. T-47D stopped proliferating 12 h after induction of wild-type *DBC2*. Growth suppression lasted for about 36 h. In contrast, induced expression of mutant *DBC2* (Asp-299 \rightarrow Asn) did not suppress growth (Fig. 6). Induced expression was confirmed by RNA blot analysis (see for example Fig. 7).

Discussion

HDs have been a good starting point for the discovery of tumor suppressor genes. Many researchers have tried to detect these HDs by comparing tumors with corresponding normal tissues (19). Arbitrarily primed PCR (APPCR) has been successfully used in some cases, but it can examine only a small portion of genome, because certain combinations of primer will amplify limited numbers of fragments. Furthermore, cloning the differences detected by APPCR is often difficult. Comparative genomic hybridization can screen the entire genome and is a powerful tool for detecting loss of heterozygosity (LOH) and amplification, but its relatively low resolution neither allows HD detection nor provides much help in localizing regions. DNA

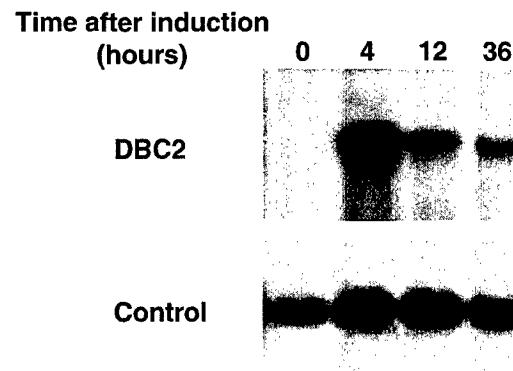


Fig. 7. RNA blot analysis of T47D with inducible *DBC2*. T-47D cells with inducible *DBC2* were treated with 5 μ M of Muristerone A. *DBC2* expression was examined at various time points after induction. The *DBC2* transcript was detected only after induction. The control is β actin.

microarray technology may be sufficiently sensitive to detect HD (20), but the true utility of this technique for identifying tumor suppressor genes remains to be established. RDA is currently an effective method to clone HD probes (3, 4), although it too has certain limitations. These limitations include the need for further analysis to distinguish HD from LOH, and that HD probes found by RDA are generally from large deletions. Hence, detailed deletion mapping is required to define the targeted genetic regions. The first problem is not insurmountable. RDA favors detection of HD over LOH, and RDA probes can be further analyzed to distinguish between these events. The second problem is likely to ease as the transcription map of humans becomes better defined.

The initial discovery of an RDA HD probe has resulted in the definition of a region on chromosome 8p that is frequently deleted in breast cancer. The HD region at 8p21, as illustrated here, contains at least six genes. In this report, we have focused on the analysis of two genes, *TNFRSF10B* and *DBC2*, because they reside in the deletion epicenter. There are four other genes that map to this region and are deleted in at least one tumor, but they are less frequently deleted. One of these, *DBC1*, was discovered by us; although it has a long ORF, it shows no homology to any known genes. It is expressed ubiquitously, but we found no point mutations within its coding domains (see Table 1). *KIAA0273*, found on the border of the region, corresponds to a spliced EST with no known function, and we do not find it expressed in normal breast tissue (data not presented). It was not studied further. *EGR3* is a transcription factor implicated in the development of neuromuscular junctions. Knockout mice are not reported to show increased tumorigenicity, and it was not studied further (21). *TNFRSF10A* encodes a decoy receptor for TNF, and we would therefore not suspect it as a tumor suppressor.

In contrast to *EGR3*, *DBC1*, *KIAA0273*, and *TNFRSF10A*, *TNFRSF10B* looked like a reasonable tumor suppressor candidate. *TNFRSF10B* encodes the Trail2 receptor for TNF, and mutations in the death domain of *TNFRSF10B* in nonsmall cell lung cancer had been previously reported. Overexpression of *TNFRSF10B* induces apoptosis of tumor cells in a *p53*-dependent manner (22). However, we did not detect mutations in *TNFRSF10B* in breast cancers, implying either that *TNFRSF10B* is not the tumor suppressor gene involved in breast cancer, or that its inactivation mechanism is not frequently caused by mutations in coding regions. We believe the latter to be unlikely, but *TNFRSF10B* cannot be completely ruled out as a candidate tumor suppressor for breast cancer.

The discovery of missense mutations in the coding region of *DBC2*, residing at the epicenter of deletion, makes *DBC2* the lead candidate for a tumor suppressor gene within the deletion locus at 8p21. Three of four mutations we discovered are located in the BTB/POZ domain and create radical amino acid substitutions likely to change protein function. In fact, functional analysis revealed that *DBC2* suppresses growth of a breast cancer cell line T-47D, but a mutant (Asp-299→Asn) does not. If we sum all deletions and point mutations, we find genetic alteration of *DBC2* in nearly 10% of breast cancer samples. Furthermore, our work indicates that, whereas *DBC2*, *TNFRSF10B*, and *DBC1* are all expressed in normal breast tissue, only expression of *DBC2* is frequently extinguished in breast cancers (11/19).

All these data suggest the involvement of *DBC2* in breast cancer, but its involvement in other cancers is not excluded. In fact, mutation in *DBC2* was found in one lung cancer cell line, and *DBC2* expression was found extinguished in lung cancers.

DBC2 expression is not suppressed in colon cancer or other types of tumors we have tested. These findings imply that *DBC2* may function as a tumor suppressor in a tissue specific manner. A possible explanation is that *DBC2* tumor suppressor function may be circumvented by alternative biological pathways in certain tissues. The existence of altered response to *DBC2* is supported by the observation that nearly half of breast cancer cells express *DBC2*. This hypothesis will also explain resistance of HeLa cells to growth inhibition caused by *DBC2* expression.

The structure of *DBC2* suggests that it encodes a signal transduction protein. It encodes a protein with significant RAS homology and contains known protein–protein interaction domains. Its novel combination of structural domains, lack of a membrane anchor sequence at the C terminus, and divergence from RAS proteins with known functions suggest that its function may be distinct from other members of the superfamily. Curiously, although *DBC2* is an evolutionarily conserved protein, with homologs *M. muculus* and *D. melanogaster*, homologs are not found in all eukaryotes and are conspicuously absent in *Caenorhabditis elegans* (23) and *Saccharomyces cerevisiae* (<http://genome-www.stanford.edu/Saccharomyces/>). Further work is clearly warranted to illuminate the role of *DBC2* in normal and pathophysiology.

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